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BPV1 E2 Protein Enhances Packaging of Full-Length Plasmid DNA in BPV1 Pseudovirions

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We studied determinants of efficient encapsidation of circular DNA, incorporating a PV early region DNA sequence (nt 584–1978) previously shown to enhance packaging of DNA within papillomavirus (PV)-like particles (VLPs). Insect coelomic cells (Sf-9) and cultured monkey kidney cells (Cos-1) were transfected with an 8-kb reporter plasmid incorporating the putative BPV packaging sequence and infected with BPV1 L1 and L2 recombinant baculovirus or vaccinia virus. Heavy (1.34 g/ml) and light (1.30 g/ml) VLPs were produced, and each packaged some of the input plasmid. In light VLPs, truncated plasmids, which nevertheless incorporated the PV-derived DNA packaging sequence, were more common than full-length plasmids. Packaging efficiency of the plasmid was estimated at 1 plasmid per 10^4 VLPs in both Cos-1 and Sf-9 cells. In each cell type, expression of the BPV1 early region protein E2 *in trans* doubled the quantity of heavy but not light VLPs and also increased the packaging efficiency of full-length circular plasmids by threefold in heavy VLPs. The resultant pseudovirions incorporated significant amounts of E2 protein. Pseudovirions, comprising plasmids packaged within heavy VLPs, mediated the delivery of packaged plasmid into Cos-1 cells, whereby "infectivity" was blocked by antisera to BPV1 L1, but not antisera to BPV1 E4. We conclude that (a) packaging of DNA within PV L1+L2 pseudovirions is enhanced by BPV1 E2 acting *in trans,* (b) E2 may be packaged with the pseudovirion, and (c) E2-mediated enhancement of packaging favors 8-kb plasmid incorporation over incorporation of shorter DNA sequences.

INTRODUCTION

Papillomaviruses (PVs) are species-specific, epitheliotropic, double-stranded DNA viruses. They are nonenveloped, 50- to 60-nm icosahedral structures (Baker *et al.,* 1991) that are composed of conserved L1 major and less conserved L2 minor capsid proteins. Studies of DNA packaging into PV-like particles (VLPs) by our laboratory (Zhou *et al.,* 1993; Zhao *et al.,* 1998, 1999) and other laboratories (Roden *et al.,* 1996; Stauffer *et al.,* 1998) have indicated that the expression of L1 and L2 capsid proteins together are essential for plasmid DNA encapsidation. For other viruses noncapsid proteins are involved in DNA packaging. For example, a protein (p40) in herpes simplex virus is strongly linked with the process of DNA packaging although it is not a major component of the infectious virus (Rixon *et al.,* 1988). Similarly, bacteriophage P22 gene 2 and 3 proteins are required for successful DNA packaging during progeny virion assembly (Casjens and King, 1974, 1975; Casjens *et al.,* 1992). In PV, five noncapsid proteins (E1, E2, E5, E6, and E7) are involved in DNA replication. Although DNA packaging studies in papillomaviruses have been extensively conducted (Zhou *et al.,* 1993; Zhao *et al.,* 1998, 1999; Roden *et al.,* 1994, 1995, 1996; Unckell *et al.,* 1997; Stauffer *et al.,* 1998; Touze and Coursaget, 1998; Kawana *et al.,* 1998), it is not clear whether any PV early protein is involved. The E2 protein of HPV plays a central role in the viral life cycle by regulating both transcription and replication of the viral genome (Desaintes *et al.,* 1997). This protein binds to a palindromic DNA sequence present in several copies in the regulatory region of all PVs (reviewed by Ham *et al.,* 1991). E2 also acts, in concert with E1 and cellular replication factors, in the initiation of viral DNA replication (Ustav and Stenlund, 1991; Chiang *et al.,* 1992a, b; Del Vecchio *et al.,* 1992). Bovine papillomavirus (BPV) type 1 has been used as a model for the study of PV replication, transcription, and cell transformation (Ustav and Stenlund, 1991; Chiang *et al.,* 1992a, b; Spalholz *et al.,* 1985) and for the study of plasmid DNA encapsidation in our laboratory (Zhou *et al.,* 1993; Zhao *et al.,* 1998, 1999). To improve our understanding of DNA packaging, we have studied the effects of two early proteins, E1 and E2, on encapsidation of plasmid by BPV L1/L2 capsids. We have also estimated the efficiency of DNA packaging and examined the characteristics of encapsidated plasmid DNA in individual VLPs. Finally, we have investigated the delivery of packaged plasmid mediated by pseudovirions into Cos-1 cells and the neutralization of the "infectivity" of pseudovirions by antisera.

RESULTS

E2 increases the numbers of VLPs with encapsidated plasmid

We have previously demonstrated that packaging of 8-kb circular DNA by PV L1 and L2 proteins produces

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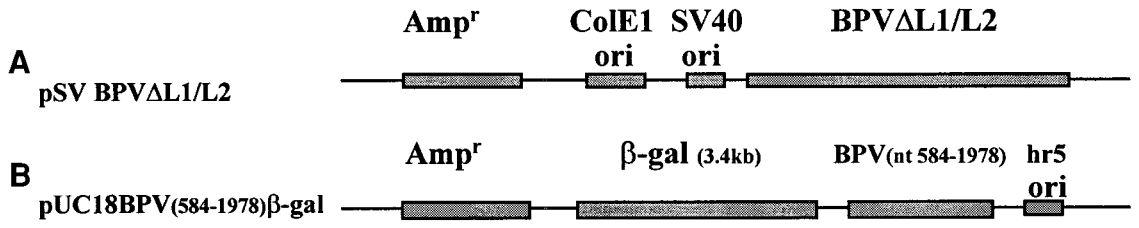

FIG. 1. Plasmids used for DNA packaging experiments. (A) pSV BPV1ΔL1/L2, used as the target plasmid for packaging experiments with PV capsid proteins expressed using L1/L2 recombinant vaccinia virus, and (B) pUC18 BPV(nt 584–1978)β-gal, used as the target plasmid in SF9 cells with L1/L2 recombinant baculovirus.

pseudovirions in cell culture and was enhanced if a 120-bp sequence from the early region of the PV genome incorporating parts of the E1 and E2 ORF was provided *in cis,* although no entire open reading frame is contained within this sequence (Zhao *et al.,* 1999). To investigate whether PV early proteins E1 and E2 provided *in trans* can alter the efficiency of DNA packaging, we first examined packaging of the plasmid pSV BPV1ΔL1/L2 in Cos-1 cells infected with BPV L1/L2 recombinant vaccinia virus (rVV), in the presence or in the absence of E1 and E2. The plasmid pSV BPV1ΔL1/L2 (Fig. 1A) is about 8 kb, comparable to the length of the PV genome, which includes most of the BPV genome except the L1/L2 gene sequence. The presence of the SV40 *ori* and ColE *ori* sequences in the plasmid allows replication in large T antigen-expressing Cos-1 cells and in bacteria, respectively. Standardized numbers of Cos-1 cells, after transfection with pSV BPV1ΔL1/L2, were infected at 5 plaque-forming units (PFU)/cell with BPV L1/L2, E1, and E2 rVVs using four different combinations: (a) rVV BPV L1/L2 alone, (b) BPV L1/L2 + BPV E1, (c) BPV L1/L2 + BPV E1/E2, and (d) BPV L1/L2 + BPV E2. VLPs were purified from a lysate of the infected cells by density gradient separation, and plasmid DNA extracted from the VLPs was used to transform *Escherichia coli.* The number of ampicillin-resistant (*AmpR*$^+$) bacterial colonies was used as a measure of packaging efficiency of the input plasmid by the BPV1 capsid proteins (Figs. 2A, 2B, and 2C). Expression of E1 protein *in trans* did not affect plasmid encapsidation by BPV VLPs (Fig. 2B). When VLPs were produced in cells expressing E2 *in trans,* packaging of input plasmid DNA was more efficient in the heavy VLP fraction (fraction 9) (Fig. 2C), which appears to be lighter than the equivalent peaks in Figs. 2A and 2B. Expression of E2 protein *in trans* did not increase the numbers of *AmpR*$^+$ colonies in light (1.30 g/ml) VLPs (Fig. 2C), suggesting that E2 protein specifically enhances full-length plasmid packaging. The effect of expression of E1 and E2 protein *in trans* on packaging was similar to that of E2 expression alone (data not shown).

To confirm the packaging findings in Cos-1 cells using the BPV1 L1/L2 rVV expression system, we have carried out further studies of DNA packaging by BPV capsids in another cell type and with another recombinant virus expression system. A plasmid, pUC18BPV (nt 584–1978)β-gal (Fig. 1B), suitable for studying DNA pack-

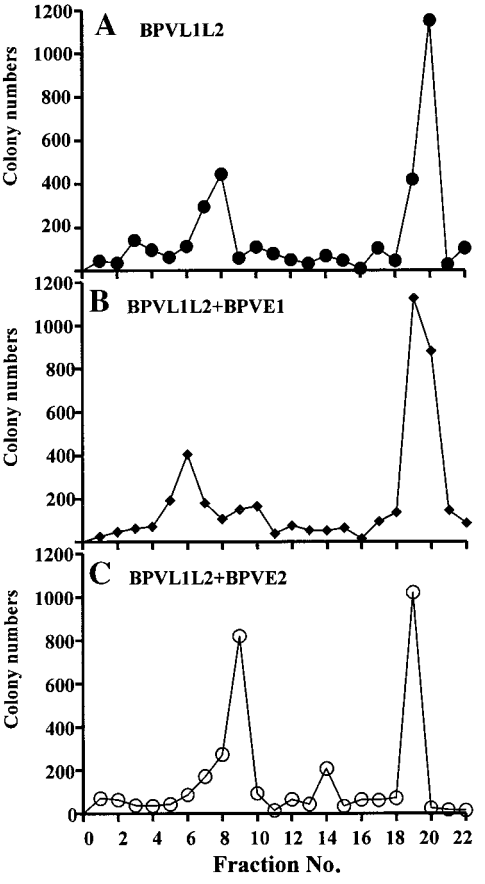

FIG. 2. Coexpression of BPV1 E2 protein increases the packaging efficacy of plasmid pSV BPV1ΔL1/L2 by BPV1 L1 and L2, expressed using rVV in Cos-1 cells. Packaging is measured as the number of ampicillin-resistant colonies obtained by transforming *E. coli* with DNA extracted from VLPs, as described under Materials and Methods. Cos-1 cells were transfected with pSV BPV1ΔL1/L2 and infected with vaccinia virus recombinant for (A) BPV1 L1/L2 alone, (B) BPV1 L1/L2 + BPV1 E1, and (C) BPV1 L1/L2 + BPV1 E2. Data points are the average of three separate experiments. Fraction 8 corresponds to a density of 1.34 g/ml and fraction 20 to 1.30 g/ml. Numbers represent fractions of CsCl gradients from bottom (heavy) to top (light). Note: Data (A) are cited from a previously published paper (Zhao *et al.,* 1998) as the comparative experiments were conducted at that time.

aging in insect cells (Sf-9 cells) was constructed, and recombinant baculovirus was used to express the PV capsid proteins. The plasmid contains a fragment (nt 1506–1625) of BPV1 recently reported to enhance DNA package into VLPs (Zhao *et al.,* 1999), while the hr5ori sequence allows plasmid replication in Sf-9 cells. Defined numbers of Sf-9 cells were transfected with this plasmid and then infected at 5 PFU/cell with recombinant baculoviruses expressing either BPV L1/L2 alone or BPV L1/L2 plus BPV E2. Plasmid DNA was extracted from purified VLPs using the same method as for Cos-1 cells, and the absolute number of plasmids was measured within a defined quantity of VLPs using real-time quantitative PCR through the detection of the *AmpR*$^+$ gene sequence as an assay of plasmid numbers. Immunoblots against L1 were used to measure total L1 protein as an indicator of VLP numbers. When BPV1 L1/L2 were expressed without E2, most VLPs obtained were of low density (1.30 g/ml) (Figs. 4A and 6A) and substantial incorporation of plasmid containing the *AmpR*$^+$ gene was observed. Heavy (1.34 g/ml) VLPs were also observed, but few of these incorporated DNA that included an intact *AmpR*$^+$ gene (Fig. 3A). When E2 protein was coexpressed with L1 and L2, the absolute amount of L1 protein in the gradient fractions incorporating heavy VLPs increased substantially (Figs. 4 and 6A), suggesting that more VLPs were packaging 8-kb DNA sequences. The amount of *AmpR*$^+$ detected in the heavier VLPs also increased threefold, indicating that E2 expression also increased the relative proportion of VLPs packaging plasmid (Figs. 3A and 3B). Expression of E2 protein *in trans* did not increase the relative proportion or absolute number of *AmpR*$^+$ DNA templates in light VLPs (Figs. 3A and 3B), further indicating that E2 protein enhances the packaging of full-length plasmid into heavy VLPs of BPV1. Overall, these results allow the conclusion that E2 enhances 8-kb DNA packaging by a specific interaction with a target DNA sequence, specific for circular DNA or for a BPV1 packaging sequence.

L1, L2, and E2 proteins are associated in VLPs

By immunoblotting, we examined the migration in a density gradient of L1, L2, and E2 proteins expressed using recombinant BVs in insect cells (Figs. 4A, 4B, and 4C) or VVs in mammalian cells (data not shown). The majority of the L1 and L2 protein was, as expected, found in the 1.30 and 1.34 g/ml gradient fractions, which had the highest numbers of VLPs visible by electron microscopy. The largest amount of packaged plasmid was also found in these fractions. These results confirm that PV L1/L2 VLPs prefer to adopt either a heavy 1.34 g/ml configuration or a light 1.30 g/ml configuration, with particles of intermediate density not favored. The distribution of L2 protein across the gradient paralleled that of L1, thus demonstrating that the different VLP densities were not due to variation in L2 incorporation into the particles (Figs. 4A and 4B). When E2 was expressed *in trans,* it was, surprisingly, found in measurable quantities in all gradient fractions containing significant amounts of VLPs (Fig. 4C). Three bands of immunoreactive E2 protein were observed in the density gradient when VLPs were assembled from BPV L1/L2 with the coexpression of BPV E2 protein, with the strongest band at 31 kDa and two faint bands at 48 and 28 kDa, respectively (Fig. 4C). Although the BPV1 DNA sequence present *in cis* in the plasmid used for packaging experiments in Cos-1 cells included an intact E2 ORF, no E2 protein was detected in the CsCl density gradient when VLPs were assembled from BPV L1/L2 only (data not shown). The data suggest that E2 protein, at least when overexpressed, may be incorporated into PV virion in association with DNA and raise the possibility that smaller amounts of E2 are incorporated under physiological conditions. The results also suggest that simple association of E2 with DNA is not sufficient to enhance packaging of longer sequences of DNA, as E2 was present in the less heavy VLP fractions.

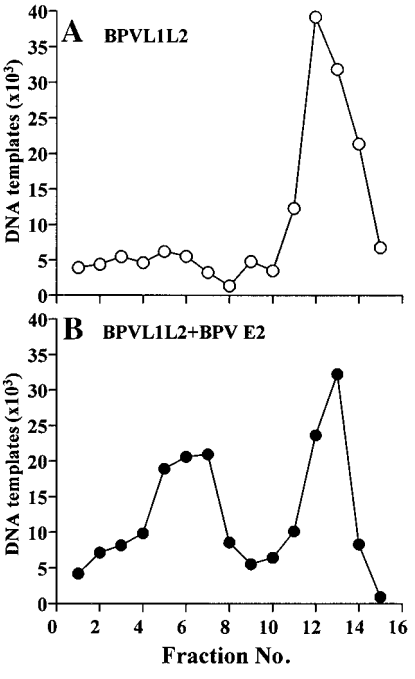

FIG. 3. Coexpression of E2 protein increases the package efficacy of plasmid pUC18 BPV(nt 584–1978)β-gal by BPV1 L1 and L2, expressed using rBV in SF-9 cells. Packaging is measured as the number of DNA templates extracted from VLPs, using real-time quantitative PCR as described under Materials and Methods. SF-9 cells were transfected with pUC18 BPV(nt 584–1978)β-gal and infected with baculovirus recombinant for (A) BPV1 L1/L2 alone and (B) BPV1 L1/L2 + BPV E2. Data points are the average of three separate experiments. Fraction 6 corresponds to a density of 1.34 g/ml and fraction 13 to 1.30 g/ml. Numbers represent fractions of CsCl gradients from bottom (heavy) to top (light).

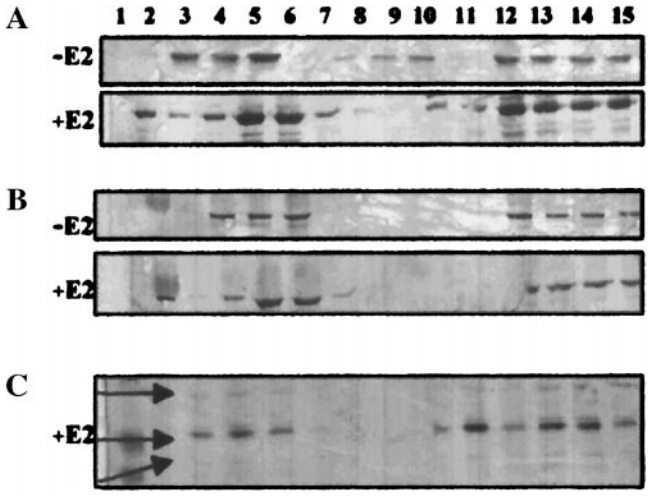

FIG. 4. Immunoblots of L1, L2, and E2 proteins in fractions from the CsCl density gradients prepared as described in the legend to Fig. 3. Numbers represent fractions of CsCl gradients from bottom (heavy) to top (light), corresponding to fraction numbers in Figs. 3A and 3B. Labeling with antibodies against L1 (A) and L2 (B) detected a single band of 55 and 77 kDa, respectively. Labeling with antibody against E2 (C) detected three bands of 28, 31, and 48 kDa (arrowed). The pUC18 BPV (nt 584–1978)β-gal-transfected Sf9 cells were infected with (−E2) recombinant baculovirus BPV1 L1/L2 only or (+E2) BPV1 L1/L2 + BPV1 E2.

E2 enhances both the production of L1 protein and the packaging efficiency of heavy VLPs

We have examined whether E2 affects the expression of BPV1 L1/L2 in Sf-9 insect cells. Total proteins were extracted from Sf-9 cells that were infected with BPV1 L1/L2 only or with BPV1 L1/L2 + BPV1 E2 without plasmid DNA transfection and used for SDS–PAGE and immunoblotting experiments (Fig. 5A). Immunoblotting reveals that both L1 and L2 were detected in protein samples from BPV recombinant baculovirus (rBV)-infected Sf-9 cells. However, L1 and L2 bands in protein samples prepared from BPV1 L1/L2 + BPV1 E2-infected Sf-9 cells were much stronger than those from Sf-9 cells infected with only BPV1 L1/L2 (Fig. 5A), indicating that E2 *in trans* enhances the production of L1 and L2 proteins in Sf-9 cells. Densitometric analysis of BPV1 L1 protein further reveals that expression of L1 and L2, without E2 *in trans,* resulted in 152.3 $\pm$ 70.8 μg of BPV L1 protein from 2×10^6 Sf-9 cells (Fig. 5B) and 370.7 $\pm$ 77.1 μg when E2 was expressed in cells *in trans* (Fig. 5B). Immunoblotting detected three bands of E2 protein of 28, 31, and 48 kDa in a sample prepared from BPV1 L1/L2 + BPV1 E2-infected Sf-9 cells, but not from BPV1 L1/L2-infected Sf-9 cells (Fig. 5A).

Immunoblotting was also used to measure the amount of L1 protein in the heavy and light fractions of a gradient prepared from Sf-9 cells infected with BPV rBVs. Expression of L1 and L2, without E2 *in trans,* resulted in 1.91 $\pm$ 0.44 μg of BPV L1 protein in heavy VLPs prepared from 2×10^6 Sf-9 cells (Fig. 6A) and 4.30 $\pm$ 1.48 μg when E2 was expressed *in trans* (Fig. 6A). No corresponding difference was seen in the amount of L1 in light VLPs expressed with or without E2 (Fig. 6A). Based on the L1 protein data, and data on the copy number of packaged plasmids in VLPs, we calculated the number of plasmid DNA templates per microgram of L1 capsid protein. There were (1.96 $\pm$ 0.34) $\times 10^6$ DNA templates/μg of L1 protein in heavy VLPs assembled without E2 protein expression *in trans* and (7.42 $\pm$ 2.31) $\times 10^6$ in light VLPs (Fig. 6B). With E2 expression *in trans,* (5.36 $\pm$ 0.58) $\times 10^6$ DNA templates/μg were observed in heavy VLPs and (5.22 $\pm$ 0.64) $\times 10^6$ DNA templates in light VLPs (Fig. 6B). The data confirmed that E2 allowed preferential packaging of plasmid sequences as longer DNA and hence shifts the distribution of packaged plasmid DNA from light to heavy VLPs.

Assuming that one packaged VLP contains one plasmid, either full size or truncated, that can produce a DNA template, by using real-time quantitative PCR assay, we calculated that, without E2 *in trans,* about 1 of 12,000 heavy VLPs, and 1 of 6000 light VLPs, carries plasmid-derived DNA (Fig. 6C). When E2 protein was provided *in trans,* about 1 of 4000 heavy VLPs carries plasmid-derived DNA, a 3-fold increase, and about 1 of 6000 light VLPs carries plasmid-derived DNA, a result unaltered by E2. Considering that the E2 *in trans* increases the production of L1 protein 2- to 3-fold, overall, expression of E2 protein *in trans* during BPV VLP assembly *in vitro* results in a 6- to 10-fold increase of incorporation of plasmid-derived DNA in heavy VLPs.

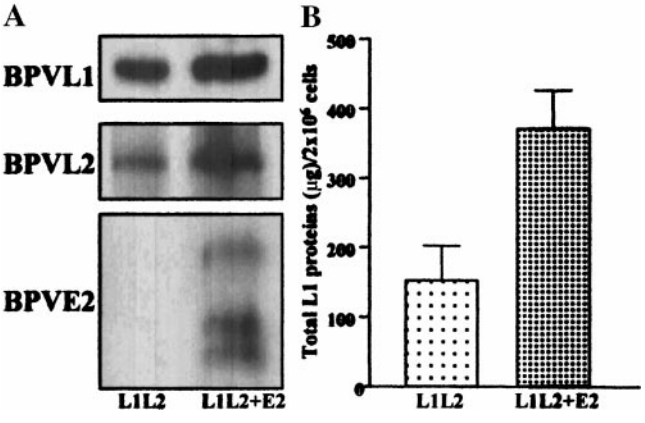

FIG. 5. Effect of E2 on expression of BPV1 L1/L2 in SF-9 insect cells. Sf-9 cells without plasmid DNA transfection were infected with BPV1 L1/L2 only or BPV1 L1/L2 + BPV1 E2. (A) Immunoblots of L1, L2, and E2 proteins extracted from Sf-9 insect cells. Labeling with antibodies against L1 and L2 detected a single band of 55 and 77 kDa, respectively. Labeling with antibody against E2 detected three bands of 28, 31, and 48 kDa. (B) Densitometric analysis of BPV1 L1 protein prepared from 2×10^6 Sf-9 cells infected with BPV rBV. Values in each histogram are the means of four independent measurements, from two separate experiments. Vertical bars indicate the standard error of the mean.

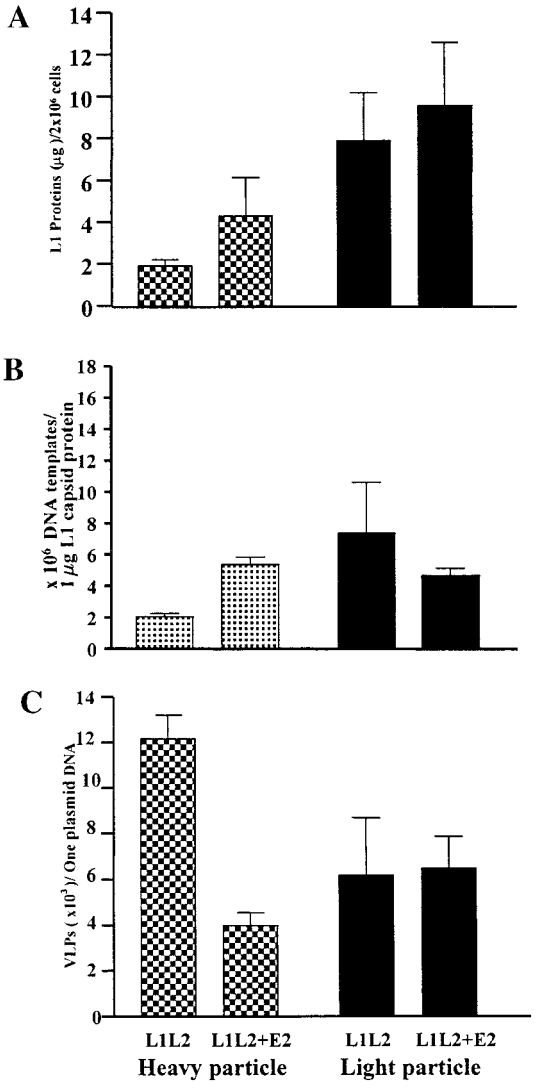

FIG. 6. Effect of E2 protein coexpression on plasmid packaging efficiency by BPV1 L1/L2 in SF-9 insect cells. Efficiency of packaging was compared for heavy (1.34 g/ml) and light (1.30 g/ml) VLPs, produced with or without E2. (A) BPV L1 protein extracted from heavy (fraction 5 in Fig. 3A and fraction 6 in Fig. 3B) and light (fraction 12 in Fig. 3A and fraction 13 in Fig. 3B) fractions of a VLP gradient prepared from 2×10^6 Sf-9 cells, measured by immunoblotting and densitometric analysis. (B) DNA templates extracted from heavy and light VLP fractions, expressed as templates per microgram of L1 protein and measured using real-time quantitative PCR as described under Materials and Methods. (C) Packaging efficiency expressed as the number of VLPs per DNA template, assuming that all VLPs contain 360 L1 molecules. Values in each histogram are the means of six independent measurements, from three separate experiments. Vertical bars indicate the standard error of the mean.

Plasmid sequences are commonly truncated in light VLPs

Previously, we have observed that truncated plasmid DNA is common in light VLPs (Zhao *et al.,* 1998). We wished to confirm whether this observation still applied when E2 protein was provided *in trans* and particularly to examine the state of a β-gal reporter construct incorporated in the packaged plasmid. Encapsidated DNA from light and heavy VLPs was characterized after transformation of *E. coli.* DNA extracted from 24 randomly selected colonies derived from each particle population was analyzed by *Hin*dIII digestion and electrophoresis. The representative results are shown in Figs. 7A and 7B. The plasmid pUC18BPV (nt 584–1978)β-gal contains two *Hin*dIII sites flanking the β-gal sequence. Eight of 24 heavy particles contained a full-length β-gal sequence and retained the flanking *Hin*dIII sites, with packaging of the full-length plasmid DNA (7.8 kb, Fig. 7B). Only 1 of 24 light particles had the full-length DNA, with the β-gal sequence (Fig. 7A). As observed previously (Zhao *et al.,* 1998), the plasmid DNA extracted from colonies derived from light VLPs was heterogeneous in size, ranging from 3.5 to 7.8 kb (Fig. 7A), with most truncated plasmids smaller than 6.6 kb.

We similarly analyzed the BPV sequence (nt 584–1978) in DNA recovered from pseudovirions produced in Sf-9 cells (Figs. 7C and 7D). Extracted DNA from 24 randomly

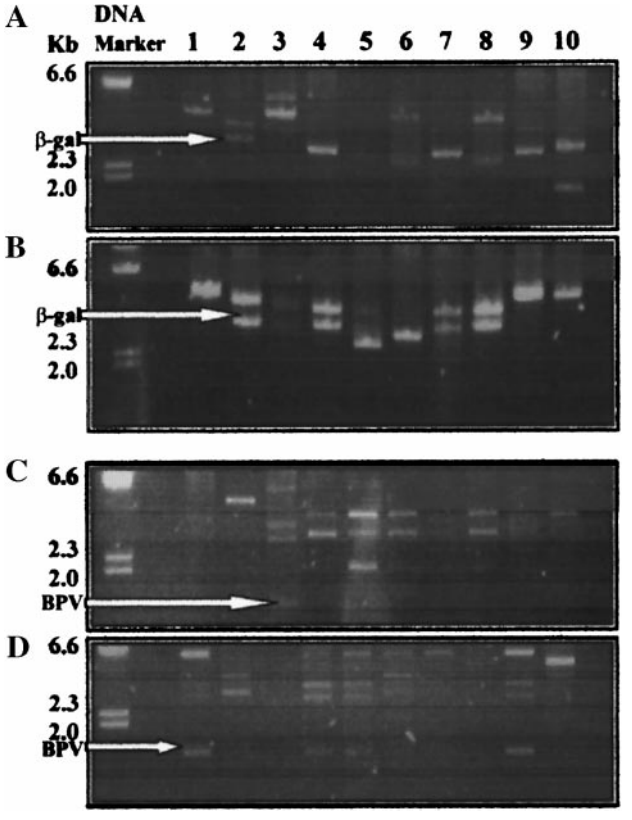

FIG. 7. Analysis of packaged plasmids recovered from heavy (1.34 g/ml) and light (1.30 g/ml) VLPs prepared in SF9 cells. Plasmid DNA was extracted from 10 random clones of bacteria transformed by DNA extracted from VLPs, digested with *Hin*dIII to detect the β-gal gene sequence (A and B) or *Xba*I to examine the BPV sequence (nt 584–1978, C and D), and electrophoresed on 1% agarose gel. (A) 1.30 g/ml VLP particles, (B) 1.34 g/ml particles, (C) 1.30 g/ml VLP particles, and (D) 1.34 g/ml particles. Lanes 1–10 are the results of randomly selected individual colonies used for enzyme restriction analysis. Solid arrows indicate a clone with the restriction pattern of the input plasmid.

selected colonies derived from each VLP population was analyzed by *Xba*I digestion and electrophoresis. Of 24 plasmids from heavy VLPs, 11 contained the BPV sequence, with packaging of the full-length plasmid (7.8 kb, Fig. 7D). In contrast, of 24 plasmids from light VLPs, only 2 contained the full-length plasmid with the BPV sequence (Fig. 7C). A high percentage of truncated plasmids produced more than two fragments after *Xba*I digestion (Figs. 7C and 7D); truncation of this plasmid commonly resulted in a new *Xba*I site, suggesting that truncation during packaging is not entirely random.

Pseudovirions can deliver packaged plasmid into Cos-1 cells

To test whether the packaged plasmid DNA could be delivered by VLPs into cells and whether the β-gal reporter gene would be expressed, 10 μl of DNase-digested VLPs was used to infect Cos-1 cells. After 52 h, cells were fixed and stained for β-gal activity. Cos-1 cells showing blue, indicative of β-gal activity, were easily visualized after exposure of the cells to heavy VLPs (Figs. 8A and 8B). The intensity of blue color among β-gal-stained cells varied from intensive to faint staining (Figs. 8A and 8B), indicating that different β-galactosidase activities accumulated in individual Cos-1 cells infected with VLPs. Only a few blue cells were visualized when Cos-1 cells were infected with light VLPs (data not shown), in keeping with the commonly truncated plasmids in light VLPs and the low packaging efficiency of full-length plasmid DNA. At the same time, we used VLPs prepared from Sf-9 cells without DNA transfection to mix with plasmid pUC18BPV (nt 584–1978)β-gal and used the VLP–plasmid mixture to infect Cos-1 cells to examine whether plasmid pUC18BPV (nt 584–1978)β-gal could be expressed in Cos-1 cells. As a result, no blue cells were visualized by X-gal staining, indicating that BPV VLPs could not mediate the transfer of unpackaged pUC18BPV (nt 584–1978)β-gal to Cos-1 cells (data not shown).

Moreover, we prepared Hirt DNA from Cos-1 cells infected with either heavy or light VLPs. PCR analysis using primers specific for β-gal and for BPV nt 584–1978 showed that both β-gal and BPV (nt 584–1978) sequences could be found in infected cells (Fig. 9B). Using β-gal and BPV (nt 584–1978) sequence probes in DNA Southern blot analysis to quantitate the amount of plasmid sequence present, however, showed that while the BPV (nt 584–1978) sequence was detected in DNA samples prepared from both heavy and light VLP-infected Cos-1 cells (Fig. 9A), the β-gal gene sequence could not be detected in DNA from light VLP-infected Cos-1 cells (Fig. 9A). This result suggests that there was selective preservation of the BPV-derived putative packaging sequence in the truncated DNA packaged within the light VLPs, confirming a specific role for this sequence in the packaging process.

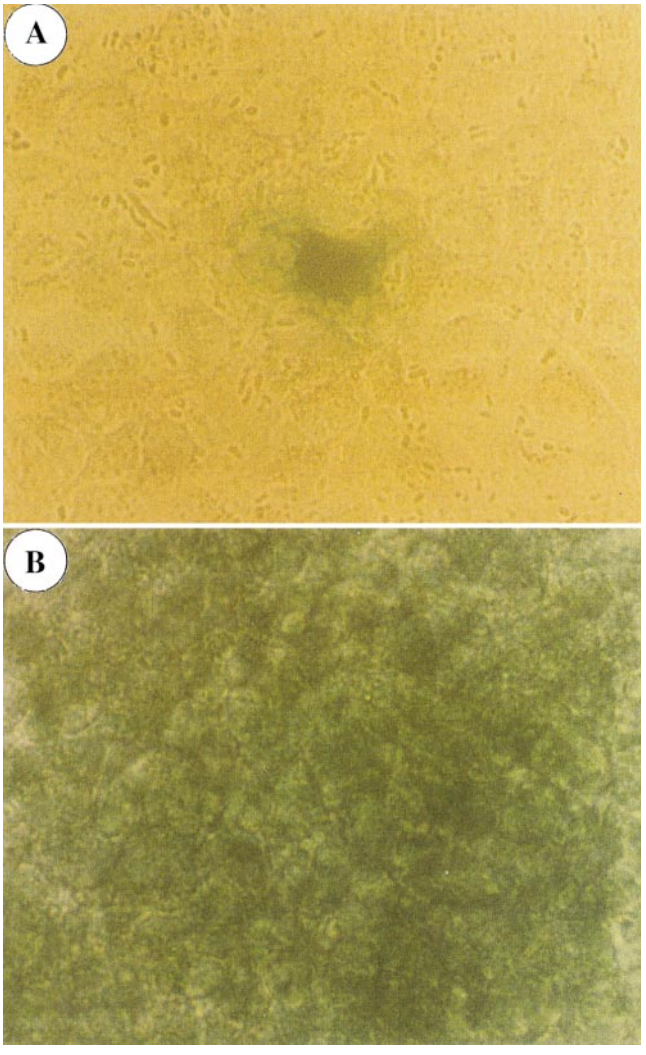

FIG. 8. Expression of β-gal after infection of Cos-1 cells with BPV1 pseudovirions containing the β-gal reporter construct. Pseudovirions (1.34 g/ml) were dialyzed against PBS containing Mg^{2+} and Ca^{2+} and digested with 5 units of DNase at 37°C for 20 min. The DNase-treated VLP suspension was incubated with Cos-1 cells, plated onto tissue culture plates, grown for 52 h, and stained with X-gal. Stained cells were examined and photographed using bright-field microscopy. (A) A single positive cell following infection at a low multiplicity of infection and (B) a large number of Cos-1 cells show strong X-gal staining after infection at a high multiplicity of infection.

Antisera neutralize the infectivity of BPV1 pseudovirions for Cos-1 cells

PV pseudovirions incorporating the β-gal reporter construct were used to examine neutralization of pseudovirion infectivity for Cos-1 cells by antibodies against BPV1 capsid protein (L1) and the early region protein (E4). Pseudovirions were exposed to antisera for 1 h prior to infection of Cos-1 cells. Antiserum to BPV1 L1 capsid was able to block infectivity of pseudovirions for Cos-1 cells, whereas antisera to E4 was not. PCR analysis of Hirt DNA from VLP-infected Cos-1 cells confirmed the neutralization results observed by staining cells for

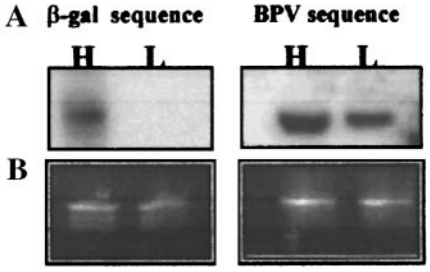

FIG. 9. Southern blot and PCR analysis of Hirt DNA prepared from Cos-1 cells infected with either heavy (1.34 g/ml) or light (1.30 g/ml) pseudovirions prepared in Sf-9 cells. Hirt DNA was examined for a β-gal-specific sequence and for the putative BPV1-derived packaging sequence. (A) Southern blot of Hirt DNA and (B) PCR for specific insert.

β-galactosidase activity (Figs. 10A and 10B). However, PCR analysis of Hirt DNA from Cos-1 cells infected with light VLPs, which were exposed to E4 antibody for 1 h, shows only a band for BPV, not for β-gal (Figs. 10A and 10B, lane 8). This probably results from the truncation of β-gal from plasmid during its packaging into light VLPs.

DISCUSSION

This study demonstrates, using an *in vitro* packaging system in insect and eukaryotic cells, that the efficacy of packaging of circular plasmid by recombinant BPV1 capsid proteins in *in vitro* systems is low and that truncation of the packaged circular plasmid is common. Where truncation occurs, a putative packaging sequence identified in previous studies (Zhao *et al.,* 1999) is preferentially preserved when compared with a β-galactosidase reporter gene. The E2 protein of BPV1, provided *in trans,* significantly improves (ca. 6- to 10-fold) the packaging efficiency of 8-kb plasmid in heavy VLPs, whereas the E1 protein does not. These results shed further light on the role of PV nonstructural proteins in the assembly of PV virions in the course of natural infection and have implications for the development of effective PV neutralization assays.

Previous studies of PV DNA packaging have demonstrated that only VLPs assembled with PV L1 and L2 capsid proteins can package the full-length BPV1 genome (Zhou *et al.,* 1993; Roden *et al.,* 1996). Some DNA is packaged within virus-like particles comprising L1 alone (Unckell *et al.,* 1997; Zhao *et al.,* 1998; Touze and Coursaget, 1998), and a significant increase in such DNA packaging occurs when the L2 protein is also available (Zhao *et al.,* 1998). L2 expression rearranges the distribution of L1 within the nucleus of the cell to protein oncogenic domains (PODs), which have been proposed as possible sites of virus assembly (Day *et al.,* 1998). One feature of *in vitro* packaging systems for PV, and, to a much lesser extent, of PV produced by natural infection, is the production of low-density light VLPs. Light VLPs are more common if L1 alone is expressed. They commonly appear empty on electron microscopic examination (Zhao *et al.,* 1998), though the present study and previous studies have shown that some DNA is incorporated with these particles (Zhao *et al.,* 1998; Touze and Coursaget, 1998), albeit of shorter length than that found in the heavier particles. The DNA packaged in light particles seems to have a preferred size of ~5 kb, and the infectivity of light VLPs, measured as successful transduction of Cos-1 cells with a reporter plasmid, was approximately 40-fold less than the infectivity of heavy VLPs. Thus, it would appear that PV L1 with or without L2 is able to package DNA up to ~5 kb and retain a buoyant density of about 1.30 g/ml. Interestingly, particles of intermediate density do not seem to be produced, at least when 8-kb circular DNA is available for packaging, suggesting that >5-kb linear DNA cannot be packaged to produce intermediate-density particles, and the production of heavy particles requires circular DNA, which presumably is packaged in a heavy supercoiled state.

A major finding of the current study is that E2 *in trans* increases the efficiency of DNA packaging. E2 is a nuclear protein containing two domains of relatively high amino acid conservation (Giri and Yaniv, 1988) and a carboxy-terminal region responsible for protein dimerization and specific DNA binding (Androphy *et al.,* 1987; Dostatni *et al.,* 1988; McBride *et al.,* 1988, 1989a). E2 activates PV DNA transcription by binding as a dimer to the E2 binding site (E2BS), a conserved sequence

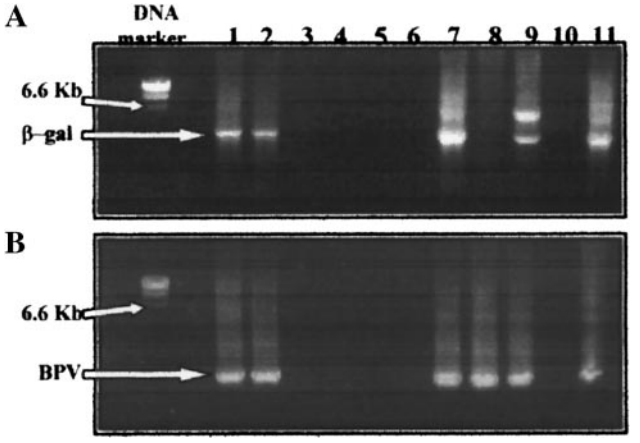

FIG. 10. Neutralization of pseudovirions by BPV-1 specific antibody. Infection of Cos-1 cells with heavy (1.34 g/ml) or light (1.30 g/ml) pseudovirions prepared in Sf-9 cells was detected by PCR analysis of DNA extracted from infected Cos-1 cells for the reporter plasmid β-gal gene sequence (A) or the putative BPV1-derived packaging sequence (B). Pseudovirions were exposed to antibody against BPV L1 capsid protein or to antibody against E4 proteins prior to infection. Lanes: (1) heavy pseudovirions without antibody; (2) light pseudovirions without antibody; (3) heavy pseudovirions with anti-L1 antibody (1:100); (4) light pseudovirions with anti-L1 antibody (1:100); (5) heavy pseudovirions with L1 antibody (1:1000); (6) light pseudovirions with L1 antibody (1:1000); (7) heavy pseudovirions with E4 antibody (1:100); (8) light pseudovirions with E4 antibody (1:100); (9) Cos-1 cells transfected with the relevant plasmid; (10) Cos-1 cells without VLP infection; (11) plasmid DNA.

(ACCGN4CGGT) (Androphy *et al.,* 1987; McBride *et al.,* 1989a; Bream *et al.,* 1993) present in multiple copies in the URR of all sequenced PV genomes. In the present study, plasmid pSV BPVΔL1/L2 contains the 17 specific E2 binding sites according to the previous report (Li *et al.,* 1989), and plasmid pUC18BPV(nt 584–1978)β-gal has two E2 binding sequences. Therefore, E2 will bind to the input plasmid DNA, though it is not possible to determine whether this binding is responsible for the observed E2-associated increase in DNA packaging in the present study. Expression of L2 causes E2 to relocate with L1 to the PODs of the cell nucleus (Day *et al.,* 1998). Thus E2 and L2 together may be involved in the association of L1 with the circular PV genome. In our system, when E2 is provided *in trans,* an increased number of particles incorporate a circular plasmid, supporting a role for E2 in linking DNA to capsid. Direct physical interaction between L2 and E2 can be demonstrated using an *in vitro* protein–protein association assay (P. Lambert, personal communication). Thus, the interaction between E2 and L2 may facilitate packaging of supercoiled DNA into PV VLPs. The overexpressed E2 appears in our system to copurify with the L1 and L2 VLPs in buoyant density gradients, as might be predicted if binding of E2 to L2 or packaged DNA is facilitating packaging. E2 is not a recognized component of naturally occurring PVs, since E2 is a low-abundance protein in a VLP gradient, making it difficult to detect in natural PV preparations. E2 expression has been found during the late stage of the viral life cycle (Burnett *et al.,* 1990; Ozbun and Meyers, 1998). Hence, in our current system the association of E2 with DNA in VLPs may not be only an artifact of protein overexpression.

According to the previous reports (McBride *et al.,* 1989b; Rank and Lambert, 1995), the BPV1 E2 open reading frame encodes three transcriptional regulatory proteins. The full-length open reading frame encodes a protein of 410 amino acids that functions as a transcriptional transactivator (E2TA). Two transcriptional repressor proteins, E2TR and E8/E2TR, contain the C-terminal 249 and 204 amino acids, respectively. In the present experiments, the plasmid that expressed E2 protein in Sf-9 cells gave rise to the E2TR species through internal ATG initiation, but not E8/E2TR. Theoretically, this plasmid could encode two E2 species; in fact, three bands of E2 proteins have been detected in both VLP fractions and total cell extracts. It is not clear where one of the smaller E2 protein species is from in our experiments. It is possible that one of the two smaller E2 proteins is the protease-resistant E2 peptide, which is degraded from full-length E2. Dostatni *et al.* (1988) reported that E2 can degrade into a protease-resistant core of 33 kDa, similar to one of the two small peptides detected in the present experiment. Therefore, further investigation is required to verify this possible explanation.

Noncapsid proteins often play a role in DNA packaging within viruses. In the bacteriophage T3 DNA packaging system (Shibata *et al.,* 1987; Morita *et al.,* 1994, 1995), two noncapsid proteins, the products of genes 18 and 19 (gp18 and gp19), aid DNA packaging into capsids by acting as molecular motors to wind the DNA into the prohead. Such packaging of phage DNA, especially *in vitro* packaging, is strongly energy-dependent (Hendrix, 1978), and the energy required for DNA packaging is provided by hydrolysis of ATP. Morita *et al.* (1994) further observed that the DNA packaging system of T3 bacteriophage displays an ATPase activity. The T3 gp19 noncapsid protein has ATP binding activity and three ATP binding and two Mg^{2+} binding consensus motifs in its amino acid sequences (Morita *et al.,* 1994, 1995). In contrast, E2 is not known to have ATPase activity, and the only PV early protein E1 with ATPase activity, E1 (Sedman and Stenlund, 1998), was not able, in our model system, to enhance DNA packaging, either alone or in concert with E2. Thus, the mechanism of E2 enhancement of DNA packaging must remain speculative, although the role of E2 interaction with its consensus binding sequence could be further studied with mutant E2 proteins lacking the DNA binding domain.

To date, *in vitro* attempts to replicate PV packaging of DNA are not very efficient—even the most efficient packaging systems apparently incorporate only 1 input plasmid per 25,000 particles (Unckell *et al.,* 1997). These figures contrast with the much higher estimates of the number of particles that will package short DNA sequences in cell-free assembly systems, where up to 40% of VLPs were high density and were assumed to have packaged DNA and up to 40% of input DNA was packaged (Touze and Coursaget, 1998). The difference is perhaps due to a lack of competing cell-derived DNA in a cell-free system. Particles derived from the cell-free system transfected target cells with an efficiency of 1:1000 "full" VLPs or 1 in 3000 input VLPs. To assess packaging efficiency in our system, we developed two independent assays to assess the efficiency of packaging of HPV DNA by the PV L1 and L2 proteins. An *E. coli* transformation assay (Zhao *et al.,* 1998, 1999) allows detection of packaged replication-competent circular plasmids that are sufficiently intact to have retained the antibiotic-resistance gene. Calculations of efficiency of packaging from this system are dependent on estimates of the efficiency of DNA extraction from the VLPs and of bacterial transduction by the input plasmid and are therefore unlikely to be precise. Real-time quantitative PCR, as pioneered by Higuchi *et al.* (1992, 1993), was therefore adapted for the current study, to obtain more accurate information about packaging efficacy. This method detects a specific fragment of the input plasmid within the $AmpR^{+}$ gene in the packaged DNA: there is no requirement for intact or circular DNA. The RT-PCR assay validated against a series of dilutions of external standard plasmids of known concentration proved suffi-

ciently reproducible to allow detection of a 30% change in input DNA concentration (data not shown). Given the propensity of PV VLPs to package truncated plasmids, these methods were complemented by analysis of packaged DNA for specific DNA sequences, including the BPV1 putative packaging sequence, which was incorporated into both plasmids, and the β-gal reporter gene. The current data allow the observation that plasmid truncation is common in light VLPs, but spare the putative packaging sequence. However, even heavy VLPs do not efficiently package the input plasmid in our system, with only 1:4000 VLPs containing the input plasmid, perhaps reflecting low input plasmid number or inefficient translocation of the input plasmid to the cell nucleus following lipofection. Alternatively, specific DNA sequences within the PV L1 and L2 genes may be required *in cis* for efficient packaging or other PV proteins besides E1, E2, L1, or L2 may be required *in trans.*

One goal for production of PV pseudovirions is the development of a system for measurement of neutralization of papillomavirus by sera from infected or immunized subjects. Notwithstanding the relatively inefficient packaging of reporter constructs, the currently described system allows production of sufficient infectious pseudovirions in insect cells to conduct virus neutralization assays and to observe transfection efficiencies of 1 cell transfected for 3000 input particles, not dissimilar to the 1 cell infected for 1000 input heavy particles reported for VLPs produced in a cell-free system (Touze and Coursaget, 1998). Further studies will address the neutralization of human PV genotypes, using the existing reporter construct and L1 and L2 proteins from different HPV genotypes to assemble pseudovirions.

MATERIALS AND METHODS

Recombinant vaccinia viruses and baculoviruses

BPV1 L1/L2 (Zhou *et al.,* 1992) rVVs, and BPV1 L1/L2 (Rose *et al.,* 1993; Paintsil *et al.,* 1996) rBVs were constructed as described previously. For construction of BPV1 E2 rVV and BPV1 E2 VBV BPV1 E2 gene sequence (nt 2608–3528) was amplified by PCR from BPV1 DNA with two oligonucleotides, 5′-GCGGATCCATGGAGACAGCATGCGAAC-3′ (BPV1 nt 2608–2626) and 5′-GCGGATCCTCACTGGTTCTTCCTCTGTG-3′ 3′ (BPV1 nt 3528–3509), which contains *Bam*HI sites (underlined). The E2 PCR product was cut with *Bam*HI and cloned into the *Bam*HI site of the RK19 plasmid to obtain RK19/BPV E2, which can express BPV E2 VVV in CV-1 cells. The E2 PCR product was also cloned into the *Bam*HI site of a baculovirus transfer vector, PVL1393, to obtain plasmid PVL1393/BPV E2, which expresses BPV E2 VBV in Sf-9 cells.

Construction of recombinant plasmids for packaging

Two plasmids (Figs. 1A and 1B) were used for DNA packaging experiments in recombinant vaccinia virus and baculovirus systems, respectively. Plasmid pSV BPVΔL1/L2 (Fig. 1A) used for DNA packaging in the vaccinia virus system was reported previously (Zhao *et al.,* 1998). Briefly, pUC18 plasmid was cut with *Bam*HI/*Hin*dIII and ligated to a 5436-base fragment with a *Bam*HI/*Hin*dIII site (nt 6958–7944/0–4450) of BPV1, which constitutes the whole BPV1 genome except L1 and L2, to obtain the pUC18 BPVΔL1/L2. Then, pSV2 *neo* was digested with *Eco*RI and *Hin*dIII, and ligated to the BPV1 genome fragment excised from pUC18BPVΔL1/L2 to the packaging plasmid pSV BPVΔL1/L2 (Fig. 1A). Plasmid pUC18BPV(nt 584–1978)β-gal (Fig. 1B) was constructed for the DNA packaging experiment in a baculovirus system. A 3.4-kb fragment of the β-gal gene sequence was amplified by PCR with two oligonucleotides, 5′-TCGAAGCTTCTCGAGGAACTGAAAAACCAG-3′ and 5′-TCGAAGCTTTTATTTTTGACACCAGACCAACTG-3′, that contained *Hin*dIII sites (underlined). A pUC18 plasmid containing hr5 ori was cut with *Hin*dIII and the *Hin*dIII-digested PCR product was inserted to produce pUC18 β-gal. Two oligonucleotides, 5′-GCTCTAGACTTAATGCTTGTGTTTCAGCTC-3′ and 5′-GCTCTAGACCTGATGCACCTGATTTCAGAC-3′, were used to amplify nt 584–1978 from BPV1 by PCR. The PCR product and the pUC18 β-gal plasmid were cut with *Xba*I and ligated to obtain the plasmid in Fig. 1B.

DNA transfection and virus infection

Plasmid pSV BPVΔL1L2 was used to transfect Cos-1 cells. Cos-1 cells, grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, CSL, Victoria, Australia), were brought up to 70% confluence in a monolayer in 150-cm^2 flasks. Cells were washed in OPTI-MEM medium (Gibco, Victoria, Australia) and transfected with 10 μg of plasmid DNA using Lipofectamine, following the instructions provided by the supplier (Gibco). After transfection, cells continued to grow in DMEM supplemented with 10% FBS. After 36 h, the transfected cells were infected with rVVs BPV L1/L2 alone, rVVs BPV L1/L2 + BPV E2, rVVs BPV L1/L2 + BPV E1, and/or rVVs BPV L1/L2 + BPV E2/E1. Infected cells were grown at 37°C for 24 h and then at 22°C for a further 24 h before use in the VLP preparation.

Plasmid pUC18BPV(nt 584–1978)β-gal was used to transfect Sf-9 cells using the calcium method. SF-9 cells, grown in Sf-9 cell medium, were brought up to 95% confluence and used for transfection in a monolayer in 150-cm^2 flasks. The transfected Sf-9 cells were incubated at 28°C for about 5 h. Then, the transfected cells were infected with either baculovirus BPV L1L2 alone or baculovirus BPV L1/L2 + BPV E2. Infected SF-9 cells

were grown at 28°C for 48 h before use in the VLP preparation.

VLP preparations and encapsidated plasmid DNA extraction

The method for preparation of VLPs from both Cos-1 and Sf-9 cells was described previously (Zhao *et al.,* 1998, 1999). The prepared VLP suspensions, after being treated with DNase (Zhao *et al.,* 1998), were used for extraction of encapsidated plasmid DNA and for the delivery and neutralization assay of encapsidated plasmid by VLPs in Cos-1 cells. Extraction of encapsidated plasmid DNA from VLPs was also described previously (Zhao *et al.,* 1998, 1999). Extracted plasmid DNA was resuspended in TE buffer and used for transformation and quantitative PCR assay.

Transformation and quantitative PCR assay of encapsidated plasmid DNA

In the vaccinia virus system, DNA was used to transform 40 μl of *E. coli* DH-α 5 cells as described previously (Zhao *et al.,* 1998, 1999). The number of antibiotic-resistant colonies was used as a measure of the efficiency of plasmid DNA packaging. In the baculovirus system, the real-time quantitative PCR assay of encapsidated plasmid DNA was adapted from methods described by Higuchi *et al.* (1992, 1993). Two oligonucleotides, 5′-CTGGATGGAGGCGGTAAATG-3′ (nt 4949–4929) and 5′-CGGCTCCAGATTTATCAGCAA-3′ (nt 4864–4885), and a probe oligonucleotide, 6FAM-CAGGACCACTTCTGCGCTCGGCTAMRA, were designed from the ampicillin-resistant gene within plasmid pUC18BPV(nt 584–1978)β-gal and used for the quantitative PCR assay.

Characterization of encapsidated plasmid DNA

The encapsidated DNA prepared from BPV VLPs assembled in the baculovirus system was used to transform *E. coli.* Randomly selected colonies were amplified in ampicillin-containing LB medium overnight. Plasmid DNA was prepared using the modified plasmid DNA minipreparation method (Zhou *et al.,* 1993). DNA pellets were resuspended in TE buffer containing RNase, restricted by *Hin*dIII or *Xba*I, and resolved on 1% agarose gel in TBE buffer.

Hirt DNA preparation

Cells were grown in DMEM supplemented with 10% FBS. Cos-1 cells, grown up to 95% confluence in monolayer in 25-cm^2 flasks, were infected with 100 μl of dialyzed VLP suspension treated with 5 units of DNase from both heavy (density of 1.34 g/ml) and light (density of about 1.30 g/ml) fractions in a CsCl gradient. Infected cells were grown at 37°C for 48 h before being used for DNA preparation. Cells were collected, pelleted by centrifugation, and resuspended in lysate buffer (10 mM Tris–HCl, pH 7.5, 10 mM EDTA, and 0.2% Triton X-100). Plasmid DNA was prepared by the Hirt method (Hirt, 1967) with some modifications (Zhao *et al.,* 1998). The extracted DNA was digested with a single enzyme (*Hin*dIII or *Xba*I) and electrophoresed on 1% agarose gel. The DNA was then blotted onto membrane and probed with either a ^{32}P-labeled β-gal probe or ^{32}P-labeled BPV (nt 584–1978) sequence probe. The extracted DNA was also used for PCR amplification using either the β-gal or the BPV sequence (nt 584–1978) oligonucleotide described above.

Immunoblotting of capsid proteins in VLP fractions

Dialyzed VLP fractions were precipitated with 10 vol of 100% ethanol at −70°C for 4 h, and the protein pellets were collected by centrifugation at 14,000 rpm for 15 min. Protein pellets, resuspended in 20 μl of 1X Laemmli buffer (Laemmli, 1970), were boiled for 5 min, separated on 10% (w/v) SDS–PAGE gels, and then electrotransferred onto nitrocellulose membranes (Bio-Rad). Blots were washed with PBS for 10 min and then blocked in PBS containing 5% nonfat milk for 1 h. Two polyclonal antibodies that react with capsid proteins L1 (Dako) and L2 (Liu *et al.,* 1997) were separately used to probe the respective capsid proteins. An antibody (kindly provided by Dr. P. Lambert, University of Wisconsin) against BPV E2 protein was also used to test for E2 protein expression. The blots were incubated with anti-rabbit secondary antibody (Silenus, Victoria, Australia), conjugated with horseradish peroxidase, at room temperature for 4 h and then stained with 3,3′-diamino-benzidine tetrahydrochloride.

Delivery and neutralization assay of encapsidated plasmid by VLPs in Cos-1 cells

Ten microliters of VLP suspensions from both heavy and light fractions was dialyzed against PBS and digested with DNase at 37°C for 30 min. For some experiments, 100 μl of DNase-digested VLP suspensions was incubated with antibodies against BPV-1 capsid protein L1 or an early region protein E4 (kindly provided by Dr. J. Doobar, National Institute for Medical Research, London, UK), diluted 1:100 or 1:1000 for 1 h. VLPs then were used to infect Cos-1 cells in a 12-well tissue culture plate. The VLP-infected Cos-1 cells, after incubation for 48 h, were fixed for β-gal staining or used for Hirt DNA preparation.

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